

# Protective Effect of Paeoniflorin on Hypoxia Reoxygenation Cardiomyocytes and its Mechanism Based on MAPK Signal Pathway

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## ABSTRACT

Mitogen-activated protein kinases (MAPK) is an important signal pathway involved in cardiomyocyte injury. To investigate the protective effect of paeoniflorin (PF) on hypoxia reoxygenation (H/R) injury and its effect on MAPK signal pathway to reveal the mechanism of PF against myocardial ischemia-reperfusion injury, in this study, the H/R model of H9C2 cells was established by hypoxia for 3 h and reoxygenation for 3 h. H9C2 cells were divided into 4 groups, the control, PF + control group, H/R group and H/R + PF group. The activities of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) were measured by colorimetry; Apoptosis rate and reactive oxygen species (ROS) content were measured by flow cytometry; the expression of Bcl-2, Caspase-3, p38 MAPK, ERK1/2 and JNK proteins were determined by WB technique. The results showed that, compared with control group, LDH, AST content, ROS level, apoptosis rate, Caspase-3, p38 MAPK, ERK1/2, JNK protein expression in H/R group were significantly increased, while cell survival rate and Bcl-2 protein expression were significantly decreased ( $P < 0.05$ ). Moreover, compared with H/R group, H/R+PF group could reduce the levels of AST, LDH, ROS ( $P < 0.05$ ), reduce the apoptosis rate and the expression of p38 MAPK, ERK1/2, JNK proteins ( $P < 0.05$ ), and increase the cell survival rate and the expression of Bcl-2 protein ( $P < 0.05$ ). In conclusion, PF can protect H9C2 cells from H/R injury, which may be related to regulating the expression of MAPK pathway.

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## Authors' Contribution

Conceptualization: S-ZQ and C-GZ.  
Methodology, formal analysis, writing review and editing: S-ZQ. Software, W-PL. Validation: W-PL, M-FY and C-YL. Investigation, writing original draft preparation: W-PL. Supervision, project administration, funding acquisition: C-GZ. All authors have read and agreed to the published version of the manuscript.

## Key words

Paeoniflorin, Hypoxia reoxygenation myocardial cells, Protective effect, MAPK signal pathway

## INTRODUCTION

Ischemic heart disease (IHD) is a heart disease characterized by myocardial ischemia, hypoxia and even necrosis (Severino *et al.*, 2018). With the acceleration of the pace of life and the increase of life pressure, its incidence rate is increasing year by year (Khan *et al.*, 2020). Unblocking the coronary arteries and restoring blood supply is the main treatment modality for IHD. However, myocardial injury often increases after blood reperfusion, which is called myocardial ischemia reperfusion injury (MIRI). The pathological mechanisms underlying the development of MIRI are complex, which are related to a variety of factors including oxidative stress, calcium overload, mitochondrial dysfunction, inflammatory response, and

apoptosis (Shen *et al.*, 2019; Luo *et al.*, 2020). Among them, Mitogen-activated protein kinases (MAPK) is an important signal pathway involved in cardiomyocyte injury (Yu *et al.*, 2015) and can be activated by cytokines, cellular stress and other stimuli to mediate different cell biological responses. MAPK signal pathway mainly includes three main pathways: Extracellular signal-regulated protein kinase (ERK), C-Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) (Guo *et al.*, 2018). It was found that activation of JNK and p38MAPK pathways drives apoptosis during MIRI, while activation of ERK1/2 inhibits apoptosis (Degirmenci *et al.*, 2020; Xu *et al.*, 2015).

Paeoniflorin (PF), a monoterpene glucoside isolated from the dried root of *Paeonia lactiflora* Pall., is an effective component of Chinese herbal Radix Paeoniae. It has multiple bioactivities, such as antioxidant, anti-tumor, hepatoprotection, neuroprotective, hypolipidemic, hypoglycemic, antithrombotic, and regulation of immune function (Li *et al.*, 2018; Ngo *et al.*, 2019; Zhang *et al.*, 2017, 2018; Zhao *et al.*, 2016; Zhu *et al.*, 2016). In recent years, it has been used in the pharmacological research of prevention and treatment of MIRI and IHD. Liu and Wei (2009) investigated the cardioprotective effects of PF on ischemic reperfused rat heart *in vitro*, and found that it could

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significantly reduce myocardial ultrastructural damage and kept the morphology and structure of myofibrils and mitochondria basically normal. [Gao \*et al.\* \(2015\)](#) concluded that PF pretreatment could exert myocardial protection on MIRI rats by inhibiting NF- $\kappa$ B signal pathway. [Wang \*et al.\* \(2016\)](#) demonstrated that PF could significantly reduce the myocardial infarction area of rats with MIRI injury, inhibit myocardial oxidative damage and inflammatory reaction. [Yousif \*et al.\* \(2020\)](#) found that the administration of PF attenuated chemokine and cytokines through up-regulation of Notch-1 and Jagged-1 activation signaling pathway leading to improved left ventricular function of MIRI mice. [Wu \*et al.\* \(2020\)](#) studied the myocardial benefit effect and mechanism of PF on MIRI in rats, and found that its protective effect on MIRI may related to reducing oxidative stress and apoptosis by inhibiting the expression of apoptosis-related signaling pathway. However, the deeper mechanism of PF in MIRI is still unclear.

Therefore, this research intends to study the protective action of PF on H9C2 cells and its effect on MAPK signal pathway by constructing the model of hypoxic/reoxygenation (H/R) damage in H9C2 cells, to investigate the protective effect and its possible mechanism of PF on myocardium, so as to provide evidence for drug development of IHD.

## MATERIALS AND METHODS

### *Drugs, reagents and main instruments*

The PF (purity  $\geq 98\%$ ) was provided by Spring and Autumn Bioengineering Co., Ltd. (Nanjing, China). Lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) test kits were provided by Jiancheng Inc. (Nanjing, China). Antibodies against Caspase-3, Tubulin, P38 MAPK, ERK1/2 were provided by Proteintech Group Inc. (Chicago, IL, USA). Antibodies against Bcl-2 was provided by ImmunoWay Biotechnology (Plano, TX, USA). Antibodies against JNK (Anti-Rabbit Antibody) was provided by Affinity Biosciences LTD (Cincinnati, OH, USA). Antibodies against  $\beta$ -actin and Horseradish peroxidase-labeled goat anti-rabbit IgG were provided by Zhongshan Jinqiao Biotechnology Co., Ltd. (Beijing, China). H9C2 cells were provided by Bena Biology (Beijing, China). BCA kit was purchased from Jiangsu Cowin Biotech Co., Ltd. (Jiangsu, China).

Epoch<sup>TM</sup> enzyme-linked immunosorbent assay reader (BioTek, Instruments, Inc., Winooski, VT, USA), Mini-PROTEAN Tetra Cell Protein Electrophoresis Transfer System (Bio-Rad Laboratories Inc, Hercules, CA, USA); IX51 Inverted fluorescence microscope (Olympus Corporation, Tokyo, Japan); ChemiScope 6100 Fluorescent chemiluminescence imaging system (Qinxiang Scientific

Instrument Co. Ltd., Shanghai, China).

### *H9C2 cell culture and preparation of hypoxia/reoxygenation (H/R) model*

H9C2 cells commonly used as an alternative for cardiomyocytes were cultured in a complete DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C in an incubator with 5% CO<sub>2</sub>.

The preparation of H/R models referred to the method described by [Su \*et al.\* \(2021\)](#). H9C2 cells were centrifuged and resuspended for counting, inoculated into 96-well plates, phosphate buffered saline (PBS) was added and incubated in a triple-gas incubator containing 5% CO<sub>2</sub> and 95% N<sub>2</sub> for 3 h. Then the medium was discarded, injected with fresh complete medium and incubated in a 5% CO<sub>2</sub> incubator for 3 h to construct an H/R model.

### *Cell counting Kit-8 (CCK-8) assay for cell viability*

To determine the cytotoxicity of paeoniflorin, H9C2 cells were separated by centrifugation, resuspended and counted. Then the cells were added to the complete DMEM medium and cultured for 24 h under normal oxygen conditions. After that, the medium was discarded and PF solutions of different concentrations (0, 25, 50, 100, 200 and 300  $\mu\text{mol/L}$ ) were added, then cultured for 24 h. The cell survival rate was determined by CCK-8 method ([Xu \*et al.\*, 2022](#)).

To screen the concentration of paeoniflorin, H9C2 cells were cultured under conventional conditions for 24 h, and then used 0, 50, 100 and 200  $\mu\text{mol/L}$  PF for 24 h. The H/R model was prepared according to method 2.2, and the cell survival rate after hypoxia reoxygenation was determined by CCK-8 method.

### *Experimental grouping and dose*

H9C2 cells were randomly divided into four groups: Control group, PF + Control group, H/R group and H/R + PF group. The Control group was incubated using complete DMEM medium in an incubator at 37°C with 5% CO<sub>2</sub>. PF + Control group was first pretreated with PF (200  $\mu\text{mol/L}$ ) for 24 h, and then replaced with complete DMEM medium and incubated for 6 h under normoxic conditions. H/R group was constructed as described in section 2.2. The H/R + PF group was pretreated with PF (200  $\mu\text{mol/L}$ ) for 24 h and the remaining treatment was the same as H/R group.

### *Measurement of AST and LDH levels*

After treatments, the cell supernatant was collected and the AST and LDH levels were measured after myocardial H/R injury according to the instructions of AST and LDH kit.

#### Flow cytometry determination of apoptosis and ROS levels

After treatments, the culture medium was collected referring to the method described by Chen *et al.* (2022) and the apoptosis assay was performed according to the instructions. Cells were trypsin digested, washed, and supernatant removed by centrifugation. 200  $\mu\text{L}$  of membrane binding solution was used to resuspend the cells, and 10  $\mu\text{L}$  of Annexin V-fluorescein isothiocyanate (FITC) dye and 5  $\mu\text{L}$  of propidium iodide (PI) dye were added, stained for 20 min, and the apoptotic rate was determined by flow cytometry.

After treatment, the intracellular ROS level was determined using the method of Chen *et al.* (2022) and ROS assay instructions. Briefly, cells were digested after trypsin digestion, ROS fluorescent probe was added, and incubated at 30°C for 20 min, and intracellular ROS content was determined by flow cytometry.

#### Determination of the expression of p38 MAPK, Bcl-2, Caspase-3, ERK1/2 and JNK proteins

After treatment, the culture medium was discarded, and the cell lysate (containing RIPA) was added into each well to lyse the cells and extract the protein. Protein concentration was determined according to the BCA kit. The proteins were denatured, loaded in equal amounts, and SDS-PAGE electrophoresis was performed, followed by transferring at a constant current of 200 mA for 120 min. Primary antibody: p38 MAPK (1:500), Bcl-2 (1:500), Caspase-3 (1:500), ERK1/2 (1:500), JNK (1:500) solution, incubation, overnight at 4°C. Secondary antibody: Horseradish enzyme-labelled goat anti-mouse IgG (1:2000) solution, incubated at room temperature for 2 h. ECL luminescent solution was added dropwise to the film and exposed in the gel imaging system. Bcl-2 and Caspase-3 used Tubulin as an internal reference and p38 MAPK, ERK1/2 and JNK used  $\beta$ -actin as an internal reference. Greyscale value was analyzed using Image J software 1.52a (National Institutes of Health, Bethesda, MD, USA).

#### Statistical analysis

Each experiment was performed at least three times. The experimental data were expressed with  $\bar{x} \pm s$ . One-way analysis of variance (ANOVA) was used for statistical analysis,  $P < 0.05$  was considered as statistical significance difference.

## RESULTS

#### Effect of PF on cell viability rate

The cell viability rate of H9C2 cultured with PF under normal and anoxic reoxygenation conditions was

tested (Fig. 1). Under the condition of normal oxygen culture (Fig. 1A), there was no significant change in cell survival rate of the PF concentration groups of 50, 100 and 200  $\mu\text{mol/L}$  ( $P > 0.05$ ), while the cell survival rate of the concentration group of 300  $\mu\text{mol/L}$  decreased significantly ( $P < 0.05$ ). The results showed that PF 300  $\mu\text{mol/L}$  group had certain cytotoxicity to H9C2 cells.

Moreover, the survival rate of H9C2 cells decreased significantly after H/R treatment (Fig. 1B) ( $P < 0.05$ ). Compared with H/R group, the cell viability rate was significantly improved in the PF concentration groups of 50, 100 and 200  $\mu\text{mol/L}$  ( $P < 0.05$ ), with the highest cell viability rate at 200  $\mu\text{mol/L}$ . Therefore, 200  $\mu\text{mol/L}$  was selected as the final dosing concentration.

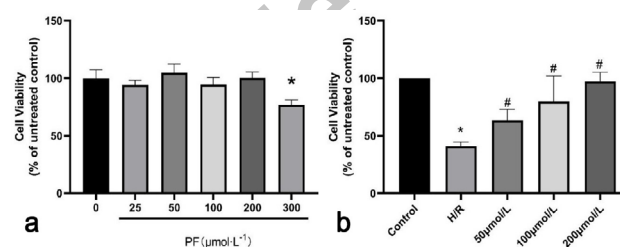


Fig. 1. Effect of different PF concentrations on H9C2 cell viability (a) under normal oxygen culture and (b) with H/R treatment.

Notes:  $\bar{x} \pm s$ ,  $n = 3$ ; Compared with 0  $\mu\text{mol/L}$ , \*  $P < 0.05$ ; Compared with H/R group, #  $P < 0.05$ .

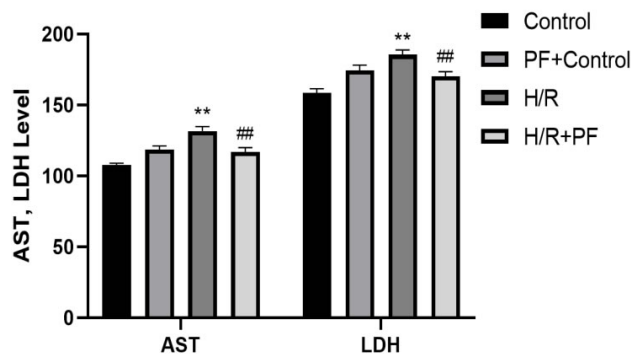


Fig. 2. Effect of PF on AST and LDH levels in H9C2 cells injured by H/R.

Notes:  $\bar{x} \pm s$ ,  $n = 3$ ; \*\*  $P < 0.01$ , compared with control group; ##  $P < 0.01$ , compared with H/R group.

#### Effect of PF on activity levels of AST and LDH

As shown in Figure 2, compared with the control group, the levels of AST and LDH in PF + Control group had no significant difference ( $P > 0.05$ ), while the levels of AST and LDH in H/R group were significantly increase ( $P < 0.01$ ). Compared with H/R group, AST and LDH levels

in H/R + PF group decreased significantly ( $P < 0.01$ ). The results indicated that PF could reduce the AST and LDH levels of H/R injury, and had a protective benefit on H9C2 cells.

#### Effect of PF on apoptosis rate of H9C2 cells injured by H/R

The results were shown in Figure 3. Compared with the Control group, the apoptosis rate in the Control + PF group had no significant difference ( $P > 0.05$ ); while the apoptosis rate of H/R group was significantly increased ( $P < 0.01$ ). Compared with H/R group, the apoptosis rate of H/R + PF group was significantly lower ( $P < 0.01$ ). The results indicated that the apoptosis rate was increased in H9C2 cells with H/R injury, while PF reduced the apoptosis rate and inhibited apoptosis.

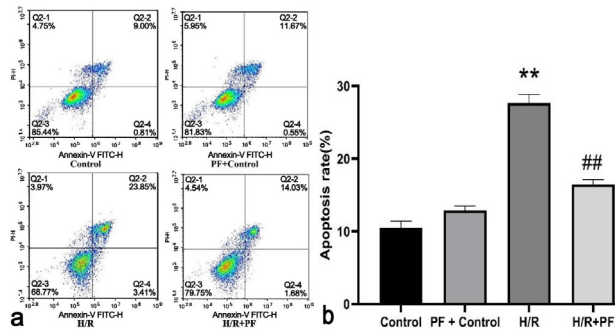


Fig. 3. Effect of PF on apoptosis rate of H9C2 cells injured by H/R.

Notes:  $\bar{x} \pm s$ ,  $n = 3$ ; \*\*  $P < 0.01$ , compared with Control group; ###  $P < 0.01$ , compared with H/R group.

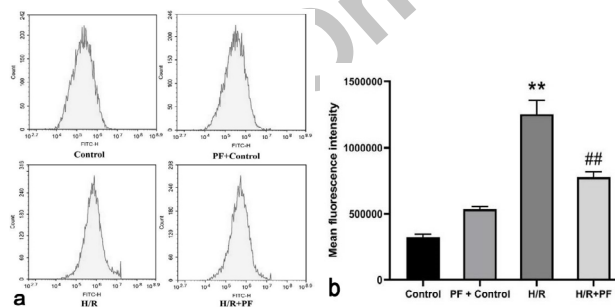


Fig. 4. Effect of PF on ROS level in H9C2 cells injured by H/R.

Notes:  $\bar{x} \pm s$ ,  $n = 3$ ; \*\*  $P < 0.01$ , compared with Control group; ###  $P < 0.01$ , compared with H/R group.

#### Effect of PF on the ROS content

It can be seen from Figure 4, compared with the Control group, the ROS content in the Control + PF group had no significant difference ( $P > 0.05$ ), while the ROS

content in the H/R group was significantly increased ( $P < 0.01$ ). Compared with H/R group, the ROS content in H/R + PF group was significantly decreased ( $P < 0.01$ ). The results showed that PF could reduce the ROS level of H9C2 cells injured by H/R.

#### Effect of PF on the expression of p38 MAPK, Bcl-2, Caspase-3, ERK1/2 and JNK proteins in H9C2 cells

It can be seen from Figure 5, compared with the Control group, the expression of Caspase-3 and Bcl-2 protein in the Control + PF group had no significant difference ( $P > 0.05$ ), but the expression of Bcl-2 protein in the H/R group decreased significantly ( $P < 0.01$ ), and the expression of Caspase-3 protein increased significantly ( $P < 0.01$ ). Compared with H/R group, the expression of Bcl-2 protein in H/R+PF group was significantly increased ( $P < 0.01$ ), and the expression of Caspase-3 protein was decreased, but there was no statistical significance ( $P > 0.05$ ). The results showed that PF could inhibit apoptosis induced by H/R injury.

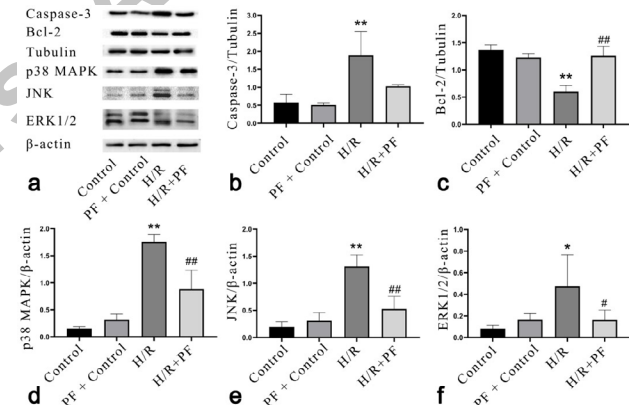


Fig. 5. Representative picture of Caspase-3, Bcl-2, p38 MAPK, ERK1/2, and JNK in H9C2 cell of each group (a), and Effect of PF on the expression of Caspase-3 (b), Bcl-2 proteins (c), p38 MAPK (d), JNK (e), and ERK1/2 (f) proteins in H9C2 cells.

Notes:  $\bar{x} \pm s$ ,  $n = 3$ ; \*  $P < 0.05$ , \*\*  $P < 0.01$ , compared with control group; #  $P < 0.05$ , ###  $P < 0.01$ , compared with H/R group.

Compared with the Control group, the expression of p38 MAPK, ERK1/2 and JNK protein in the Control + PF group had no significant difference ( $P > 0.05$ ), while the expression of p38 MAPK, ERK1/2 and JNK protein in the H/R group increased significantly ( $P < 0.05$ ). Compared with H/R group, the expression of p38 MAPK, ERK1/2 and JNK protein in H/R + PF group decreased significantly ( $P < 0.05$ ). The results indicated that PF had some regulatory effects on the MAPK signaling pathway.

## DISCUSSION

MIRI is a secondary injury resulting from myocardial ischemia-reperfusion, which severely reduces the outcome of ischemic heart disease. PF is an effective component extracted from Chinese herbal paeonia lactiflora, which has a variety of biological activities. It was found that PF has the effects of reducing myocardial fibrosis in rats with heart failure (Liu *et al.*, 2020), decreasing the infarct area of MIRI (Chen *et al.*, 2018a), and protecting vascular endothelial cells (Wang *et al.*, 2019). Chen *et al.* (2015) developed that PF can improve the expression of caspase-3 and caspase-9, inhibit inflammation, and play a protective role in infarcted myocardium, which has certain research value in the treatment of cardiovascular diseases.

LDH and AST are enzymes distributed in myocardial tissue and are routinely tested to reflect myocardial injury (Abdul *et al.*, 2015). LDH and AST are released from myocardial tissue into the blood after myocardial damage, resulting in their elevated levels in the serum (Chen *et al.*, 2012). The results in this study indicated that H9C2 cardiomyocytes could cause a significant increase in both LDH and AST levels after H/R injury. LDH and AST levels were reduced after administration via PF. It is suggested that PF can protect H9C2 cardiomyocytes from H/R injury.

The occurrence of MIRI is related to several factors, among which oxidative stress is one of the main causes (Cadenas, 2018). It has been found that during MIRI, a large amount of ROS is generated instantaneously, and the excess ROS will damage the cell membrane and induce apoptosis in cardiomyocytes (Chen *et al.*, 2018b). Apoptosis is regulated by a variety of genes, among which *Bcl-2* is an anti-apoptotic gene, *Caspase-3* is a pro-apoptotic molecule as well as a marker gene for apoptosis (Lee *et al.*, 2007). Xie *et al.* (2001) found that MIRI reduced the expression of *Bcl-2* in cardiomyocytes with a pro-apoptotic tendency. Chen *et al.* (2001) found that *Bcl-2* overexpressing transgenic mice were able to inhibit apoptosis and attenuate MIRI. Liu (2014) found that interference with *Caspase-3* expression reduced apoptosis of cardiomyocytes and improved cardiac function. This study found that after injured by H/R in H9C2 cells, ROS activity was significantly increased, apoptosis rate was increased, *Bcl-2* protein expression level was decreased, while *Caspase-3* protein expression was increased, indicating that oxidative stress injury occurred after H/R and induced apoptosis in cells. PF was able to reduce ROS levels, decrease *Caspase-3* protein expression, increase *Bcl-2* protein expression, and inhibit apoptosis.

Apoptosis occurs with the involvement of multiple signaling pathways, of which the MAPK pathway is an

important regulatory pathway. p38 MAPK, JNK and ERK play important roles in the process of MIRI (Yu *et al.*, 2015). It was found that MIRI causes upregulation of p38 MAPK, JNK, ERK expression and their phosphorylation levels in MAPK pathway, however, reducing the expression of MAPK pathway can help to reduce the damage caused by MIRI (Hsiao *et al.*, 2021). This study shows that PF can reduce the expression of p38 MAPK, JNK, and ERK1/2 proteins in the MAPK pathway, indicating that PF may be able to protect H9C2 cells by regulating the MAPK pathway.

## CONCLUSION

In summary, H9C2 cardiomyocytes caused peroxidative damage to cardiomyocytes after hypoxic-reoxygenation, release of cardiac enzymes, and induction of apoptosis. PF can reduce LDH and AST levels, resist oxidative stress damage, inhibit apoptosis and protect cardiomyocytes. Its anti-apoptotic effect may be related to the regulation of MAPK pathway.

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### IRB approval

This study did not involve human or animals.

### Ethical statement

There are no studies on animals or humans, only cultured cells are involved.

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